



## Review

## Emerging concepts in the therapy of mitochondrial disease

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## ABSTRACT

Mitochondrial disorders are an important group of genetic conditions characterized by impaired oxidative phosphorylation. Mitochondrial disorders come with an impressive variability of symptoms, organ involvement, and clinical course, which considerably impact the quality of life and quite often shorten the lifespan expectancy. Although the last 20 years have witnessed an exponential increase in understanding the genetic and biochemical mechanisms leading to disease, this has not resulted in the development of effective therapeutic approaches, amenable of improving clinical course and outcome of these conditions to any significant extent. Therapeutic options for mitochondrial diseases still remain focused on supportive interventions aimed at relieving complications. However, new therapeutic strategies have recently been emerging, some of which have shown potential efficacy at the pre-clinical level. This review will present the state of the art on experimental therapy for mitochondrial disorders.

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## 1. Introduction

## 1.1. Basic concepts of mitochondrial biology and medicine

Mitochondria are semi-autonomous double-membrane organelles, the inner membrane being folded to form mitochondrial cristae, where respiratory chain (RC) complexes reside.

The main role of mitochondria is to extract energy from nutrients through respiration, and convert it into heat, or store it as ATP, the energy currency of cells. This is ultimately carried out by the respiratory chain (RC), through a process termed oxidative phosphorylation (OXPHOS). Respiration is performed by four multiheteromeric RC complexes, CI–IV, that transfer the electrons stripped off from nutrient-derived substrates as hydrogen atoms, to molecular oxygen. Electrons are conveyed to the RC through redox shuttle moieties, NADH + H<sup>+</sup> for complex I, FADH<sub>2</sub> for complex II. This electron flow is coupled with the translocation of protons across the inner mitochondrial membrane from the matrix to the intermembrane space, operated by complexes I, III and IV, generating an electrochemical gradient which is then exploited by RC complex V (or ATP synthase) to carry out the condensation of ADP and Pi into ATP [1].

Mitochondria have their own DNA (mtDNA), a maternally inherited, double-stranded circular molecule of 16.5 kb in mammals, encoding 13

subunits of the RC complexes I, III, IV and V (complex II is composed of four nucleus-encoded subunit with no contribution from mtDNA). In addition, mtDNA contains 22 tRNAs, and 2 rRNA genes, which form the RNA apparatus serving the in situ translation of the 13 mtDNA-encoded respiratory chain subunits. MtDNA is present in hundreds to thousands of copies in the different cell types in an individual. In normal individuals, mtDNAs are all identical to each other, a condition termed homoplasmy. However, pathogenic mtDNA mutations are frequently co-existing in variable amount with wild-type mtDNA molecules, a condition termed heteroplasmy. The rest of the mitochondrial proteome, which is estimated to consist of approximately 1500 polypeptides, is encoded by nuclear genes, translated in the cytosol into proteins, which are eventually targeted to and imported into the organelles by an active process.

Complex I (NADH-ubiquinone oxidoreductase) contains seven mtDNA-encoded subunits (ND1–ND6 and ND4L) and at least 37 nucleus-encoded subunits of complex I; electrons are transferred from NADH, the main redox shuttle of pyruvate dehydrogenase and TCA cycle, onto a hydrophobic mobile electron carrier, ubiquinone (coenzyme Q, CoQ). Complex II (succinate-ubiquinone oxidoreductase) is composed of only four subunits, all encoded by the nuclear genome and transfers electrons from FADH<sub>2</sub>, mainly derived from beta-oxidation of fatty acids, to CoQ. Complex III (ubiquinol-ferricytochrome c oxidoreductase) has a single mtDNA-encoded subunit, apocytochrome b, and 10 subunits encoded by the nuclear genome. Complex III transfers electrons from CoQ to another electron shuttle, cytochrome c, which in turn transfers them to complex IV. Complex IV (cytochrome c oxidase, COX), which is composed of three mtDNA-encoded and 11 nucleus-encoded subunits,

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transfers electrons to molecular oxygen, with the formation of water. Complex V (oligomycin-sensitive ATP synthase), which utilizes the energy potential of the electrochemical gradient to carry out ATP synthesis, is composed of two mtDNA-encoded subunits (ATPase 6 and 8), and at least 13 nuclear DNA-encoded subunits. These subunits are arranged to form two distinct particles. The membrane-embedded F<sub>0</sub> particle constitutes a rotor operated by protons flowing through it. The rotation of this structure is transmitted to the matrix-protruding F<sub>1</sub> particle, which catalyzes the biosynthesis of ATP [2].

Numerous specific assembly factors and chaperons are needed to assemble the protein backbone, insert suitable prosthetic groups and metal-containing reactive centers and form each holocomplex [3].

Other components of the mitochondrial proteome are required for a huge array of biological processes, including replication, transcription, and translation of the mtDNA, formation and assembly of the respiratory chain complexes, fission–fusion of the mitochondrial network, signaling and execution pathways (e.g. ROS production and apoptosis), scavenging of toxic compounds, and many other metabolic processes, as diverse as fatty acid oxidation, biosynthesis of pyrimidines, heme, and Fe–S clusters, etc.

From a genetic standpoint, primary mitochondrial diseases can be classified into two major categories, depending on which genome, mitochondrial or nuclear, carries the responsible mutations. MtDNA mutations include point mutations, either homo- or heteroplasmic, and (invariably heteroplasmic) large-scale rearrangements. Heteroplasmic point mutations have been found in all mitochondrial genes, and lead to different clinical phenotypes, including some canonical syndromes such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) [4], myoclonic epilepsy with ragged red fibers (MERRF) [5], neurogenic weakness, ataxia and retinitis pigmentosa (NARP) [6], and Leigh syndrome (LS). The main disease entity associated with homoplasmic mtDNA mutations is Leber's hereditary optic neuropathy (LHON) [7]. Rearrangements (single deletions or duplications) of mtDNA are responsible for sporadic progressive external ophthalmoplegia (PEO) [8], Kearns–Sayre syndrome (KSS) [8], and Pearson's syndrome [9].

Nuclear mutations have been found in a huge number of genes directly or indirectly related to the respiratory chain, encoding, for instance, (i) proteins involved in mtDNA maintenance and/or replication machinery; (ii) structural subunits of the respiratory chain complexes; (iii) assembly factors of the respiratory complexes; (iv) components of the translation apparatus; and (v) proteins of the execution pathways, such as fission/fusion and apoptosis (see [10] for an exhaustive list).

Mitochondrial diseases are hallmarked by huge clinical, biochemical and genetic heterogeneity, which hampers the collection of homogeneous cohorts of patients to establish the efficacy of a treatment. For instance, clinical outcomes in primary coenzyme Q deficiency span from encephalomyopathy, multisystem disease, cerebellar ataxia, isolated myopathy and nephrotic syndrome [11]. For unknown reasons only 20% of the patients respond to CoQ<sub>10</sub>, the only available therapy [11]. Studies in cellular models suggest that the slow pharmacokinetics of CoQ<sub>10</sub> can explain the different responses observed in humans, but more studies are needed to clarify this issue. Similarly, riboflavin is effective in some cases of mitochondrial disease due to mutations in genes encoding FMN- or FAD-dependent proteins such as NDUVF1 (the FMN binding subunit of complex I), AIFM1, ACAD9 [12,13], and SDHA (the FAD binding subunit of complex II). However, not all patients respond to riboflavin supplementation [14].

## 1.2. Experimental therapeutic strategies

Remarkable progress has been made in recent years on understanding both the fundamental pathogenic processes underlying mitochondrial disease, and the mechanisms of mitochondrial biogenesis and signaling. Based on this knowledge, sensible

therapeutic strategies have recently been proposed to combat mitochondrial disorders, for which experimental evidence is accumulating in cellular and animal models. These can be broadly divided in “generalist” strategies, which could in principle be applied to a wide spectrum of different disease conditions, and “disease-tailored” strategies, applicable to a single disease (Table 1). The first group includes: (i) regulation/activation of mitochondrial biogenesis; (ii) regulation/activation of mitochondrial autophagy; (iii) inhibition of mitochondrial apoptosis; (iv) scavenging of toxic compounds; (v) bypass of electron transfer chain defects; and (vi) nuclear transfer. The second group includes (i) scavenging of specific toxic compounds in specific diseases, (ii) supplementation of nucleotides, and (iii) gene- and cell-replacement therapies. Each of these strategies can be pursued by different approaches, such as pharmacological treatments, gene transfer to express the missing or a therapeutic protein, stem-cell/organ transplantation. This review will focus on emerging experimental (i.e. pre-clinical) therapies for mitochondrial disease. Ongoing clinical trials have recently been reviewed elsewhere [15].

## 2. Pharmacological and metabolic interventions

### 2.1. Increasing mitochondrial biogenesis

Mitochondrial diseases are hallmarked by bioenergetics defects, ultimately leading to decreased ATP synthesis. Thus, therapeutic interventions aimed at increasing the ATP levels available to cells may be beneficial. Importantly, mitochondrial disease become manifest when the residual activity of the defective gene product, either mitochondrial or nuclear encoded, falls below a critical threshold, suggesting that even partial restoration of the activity may be sufficient to rescue or at least ameliorate the phenotype. The idea that mitochondrial biogenesis is critical to determine the phenotypic outcome of disease has been boosted by the recent observation that increased mitochondrial content protects non-manifesting carriers of the LHON mutations. This can partly explain the incomplete penetrance of the disease and opens the possibility to stimulate mitochondrial biogenesis as a therapeutic strategy for LHON [16].

Increased mitochondrial biogenesis is a physiological response to stress conditions (e.g.: cold, exercise, nutritional status), which is activated to meet the energetic requirements of tissues [17].

The pathways controlling mitochondrial biogenesis (Fig. 1) have mainly been investigated in skeletal muscle and brown adipose tissue, and shown to rely, in most of the cases, on the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) coactivators 1 $\alpha$  and  $\beta$  (the PGC family). PGC proteins interact with and activate

**Table 1**  
Summary of the experimental therapies for mitochondrial diseases.

	Strategy	Method
Generalist	• Activation of mitochondrial biogenesis	Pharmacology
	• Modulation of autophagy	
	• Inhibition of apoptosis	
	• Scavenging of ROS	
	• Endurance training	
	• Dietary manipulation	AAV-mediated gene therapy
	• By-passing RC block	
	• ZFNs or TALENs to shift heteroplasmy	
	• Overexpressing aaRSs to stabilize mutated mt-tRNA	
	• Somatic nuclear transfer	
Disease-tailored	• Scavenging of toxic compounds	Pharmacology
	• Supplementation of nucleotides	
	• Replacement of the missing gene	AAV-mediated gene therapy

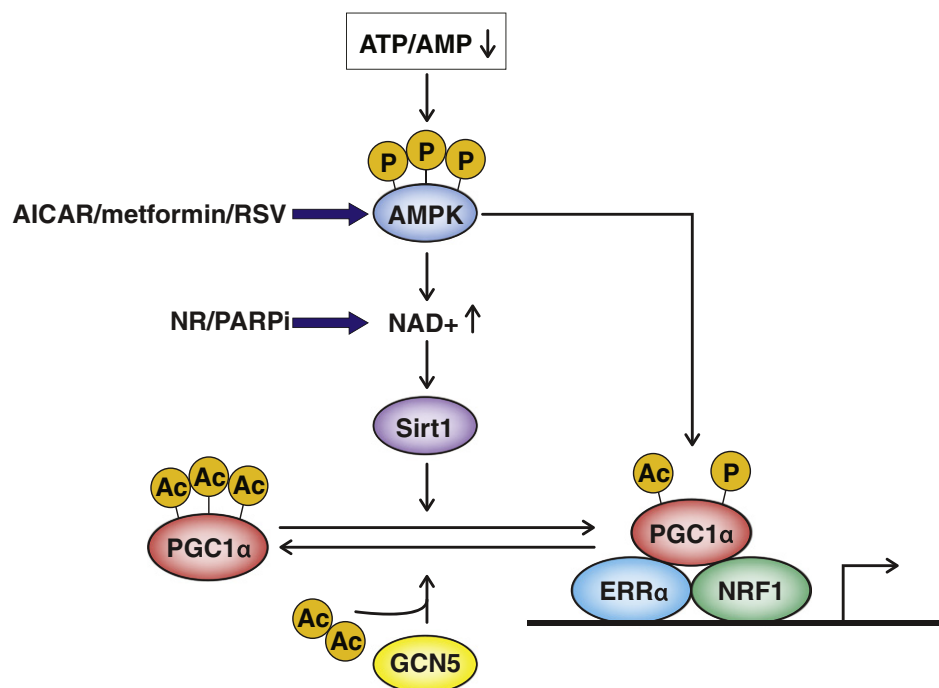


Fig. 1. PGC1 $\alpha$ -dependent mitochondrial pathway and its pharmacological modulation.

several transcription factors, including the Nuclear Respiratory Factors (NRF1 and 2), and the Peroxisomal Proliferator Activator receptors (PPAR  $\alpha$ ,  $\beta$ , and  $\gamma$ ) among others. NRFs and PPARs in turn increase the transcription of genes related to oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) pathways, respectively. PGC-1 $\alpha$  is the best characterized PGC protein. Its activity is inhibited by acetylation, which in turn is controlled by the acetylase GCN5 and deacetylase SIRT1, and is increased by phosphorylation, which depends on the activities of several kinases, including p38 MAPK, glycogen synthase kinase 3b (GSK3b) and AMP-dependent kinase (AMPK) [18,19]. Importantly, as AMPK and SIRT1 are druggable enzymes, they have been exploited in several pre-clinical experiments to activate PGC-1 $\alpha$  and induce mitochondrial biogenesis.

The idea that increasing the amount and/or function of mitochondria could be beneficial in mitochondrial disease, was tested by treating fibroblasts from patients with different mitochondrial diseases with bezafibrate [19], a pan-PPAR agonist widely used to treat metabolic syndrome and diabetes. This treatment led to an improvement in the defective activities of the respiratory chain complexes, dependent on the induction of PGC-1 $\alpha$  activity. These findings were subsequently reinforced by in vivo observations reported by Wenz et al. [20], who used both a muscle-specific PGC-1 $\alpha$  transgenic mouse and bezafibrate to improve the motor performance of a muscle-specific knockout mouse for *Cox10*, a farnesyltransferase involved in the biosynthesis of COX-specific heme *a*. Notably, this effect was not due to restoration of COX activity in isolated mitochondria but to increased mitochondrial content, which determined an overall increase in ATP availability in the muscle fibers. It is unclear why the stimulation of mitochondrial biogenesis caused by genetic or pharmacological induction of PGC-1 $\alpha$  seems more effective than that spontaneously occurring in pathological conditions such as for instance, ragged-red fibers. One hypothesis is that while the mitochondrial biogenic pathway is activated only in highly mutated, bioenergetically spent mitochondria clustering in ragged red fibers, the mitochondrial biogenetic activation through pharmacological modulation of PGC-1 $\alpha$  is

generalized and involves also OXPHOS proficient mitochondria, which can then exert effective functional complementation along the entire muscle fiber [20]. A second beneficial effect of PGC-1 $\alpha$  activation is the switch towards oxidative fiber types, which increases the energetic efficiency of the tissue [21].

Beneficial effects of bezafibrate were also reported in cybrids harboring pathological tRNA mutations [22] and in the nervous tissue of a brain-specific *Cox10* knockout mouse [23]. However, these results failed to be confirmed in subsequent studies, including bezafibrate treatment of three mouse models of COX deficiency [24–26].

The reason(s) of these discrepant results are unclear. PGC-1 $\alpha$  seems to act predominantly upstream of the PPAR receptors, i.e. as a co-activator of the PPAR-dependent pathways, rather than being induced by PPARs, although work in a reporter-gene system in cultured cells has shown that overexpressed PPARs can indeed bind to a PPAR-responsive element in the promoter of PGC-1 $\alpha$  gene, and increase the reporter gene transcription [27]. However, these results are based on highly engineered recombinant systems in cells, and have never been confirmed in animal models [28–30].

An alternative pathway to induce PGC-1 $\alpha$  dependent mitochondrial biogenesis is centered on the activation of the AMP-dependent kinase (AMPK). By using the AMPK agonist AICAR Viscomi et al. obtained robust induction of OXPHOS-related gene transcription and increase of respiratory chain complex activities in three models of COX deficiency, a *Surf1* constitutive knockout mouse (*Surf1*<sup>−/−</sup>), a *Sco2* knockout/knockin (*Sco2*<sup>KOKI</sup>) mouse and a muscle-specific *Cox15* (*ACTA-Cox15*<sup>−/−</sup>) mouse [24]. The increase in the respiratory chain activities resulted in striking improvement of motor endurance in the *Sco2*<sup>KOKI</sup>, but not in *ACTA-Cox15*<sup>−/−</sup> mice. This difference is likely related to the more severe clinical phenotype of the *ACTA-Cox15*<sup>−/−</sup> mouse model, which could not be corrected in spite of a clear, albeit partial, rescue of COX activity. In keeping with this, we observed a partial but transient increase of the motor performance in muscle-specific KO mice when the treatment with AICAR was started early during the disease course, i.e. at 4 weeks of age. Notably, *ACTA-Cox15*<sup>−/−</sup> mice overexpressing PGC-1 $\alpha$  (*PGC-1 $\alpha$* <sup>−/−</sup>) also showed improved motor performance compared to naive *ACTA*-

*Cox15*<sup>−/−</sup> littermates, but this effect was transient and, at 6 months of age, both *ACTA-Cox15*<sup>−/−</sup> and *ACTA-Cox15*<sup>−/−</sup>–*PGC-1α*<sup>−/−</sup> mice were able to run only for a few minutes on a treadmill, suggesting that *PGC-1α* delayed, but did not arrest, the disease progression (Bottani et al., unpublished). Interestingly, Saada and colleagues [31] found that AICAR was the most effective compound in inducing mitochondrial biogenesis in complex I deficient cells, whereas bezafibrate gave erratic results, as already reported by Bastin et al. [19] and also observed by us in either mutant cells (Bottani et al., unpublished) or mouse models [24]. A single report showed that bezafibrate can rescue the COX-defect of *SCO2* mutant fibroblasts [32]. However, analysis of OxPhos activities and Seahorse oxygen consumption carried out in our lab failed to show any beneficial effect of bezafibrate on a fibroblast cell line from a *SCO2* mutant patient and in *SCO2*<sup>KOKI</sup> MEFs (Bottani et al., unpublished). Finally, Bastin et al. [33] reported an increase of OXPHOS markers in bezafibrate-treated CPT2-mutant patients, but no evidence of efficacy in mitochondrial disease patients has so far been reported.

A further strategy to activate *PGC-1α* is to promote its deacetylation via Sirtuin 1 (Sirt1). Sirt1 is a nuclear deacetylase that utilizes the NAD<sup>+</sup> moiety to deacetylate acetyl-lysine residues of proteins. Notably, NAD<sup>+</sup> exerts a substrate-dependent activation of Sirt1, which has homeostatic significance, setting up mitochondrial biogenesis to NAD<sup>+</sup> availability. We recently showed that the NAD<sup>+</sup> pool can be increased by diet supplementation with its natural precursor nicotinamide riboside (NR) or by genetic or pharmacological inhibition of poly(ADP) ribosyl-polymerase 1 (Parp1), a NAD<sup>+</sup> consumer and Sirt1 competitor. These treatments lead to activation of Sirt1 (and other sirtuins) and boost mitochondrial respiration by inducing OXPHOS genes via the *PGC-1α* axis [34]. As a result, *SCO2*<sup>KOKI</sup> mice showed improved motor performance up to normal values. NR was also effective in delaying the disease progression of the *deletor* mouse, another model of mitochondrial myopathy due to expression of a mutant variant of Twinkle, the mtDNA helicase. Also in this model, NR induced robust mitochondrial biogenesis, corrected abnormalities of mitochondrial ultrastructure, and prevented the generation of multiple mtDNA rearrangements [35]. Importantly, both studies showed that NR also induced the mitochondrial unfolded protein response (UPR<sup>mt</sup>). UPR<sup>mt</sup> is a stress response that activates transcription of mitochondrial chaperones to preserve protein homeostasis within the organelle (see [36–38] for extensive review). These observations suggest the involvement of UPR<sup>mt</sup> in the protective effects provided by NR. In addition, we found that PARP-inhibitors partially improved COX deficiency also in the brain, raising the possibility for their use to target neurological defects.

Taken together these findings open the exciting perspective to use a single therapeutic strategy to target a wide spectrum of genetically heterogeneous mitochondrial diseases. However, more work is warranted to refine and optimize the most effective strategies. For instance, bezafibrate gave highly variable and poorly reproducible results, and AICAR, although highly effective and widely used in experimental work, has a short half-life after intravenous administration (1.4–2.2 h), poor bioavailability after oral ingestion (less than 5%) and causes increased blood levels of lactic acid and uric acid, making it a poor candidate for long-term use [39,40].

Conversely, the possibility to transfer into clinical practice the supplementation with NR (or other NAD<sup>+</sup> precursors) and the administration of PARP inhibitors (PARPi), seem to be more realistic options. NR is a natural compound, in fact part of vitamin B3, enriched in maternal milk, and several PARPis are currently under clinical trial as anticancer therapeutic agents [41]. However, so far the effects of these compounds have been investigated in a limited number of mouse models of mitochondrial disease. Longer-term treatments, and studies in different disease models are needed to confirm efficacy and prompt their use in clinical trials. In addition, the potential mutagenic effects of PARPis in non-cancer patients are still to be adequately investigated, although a long-term study in mouse models of diet-induced obesity, and data in patients treated with Olaparib (AZD-2281) [42], both suggest limited genomic toxicity [43].

Resveratrol (RSV) is another compound reported to trigger mitochondrial biogenesis in several animal models, including *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus*. The mechanism by which RSV activates mitochondrial biogenesis is still debated. The common idea that RSV operates via direct activation of Sirt1 has been recently challenged by showing that in fact RSV inhibits phosphodiesterase IV [44]. The consequent raise in cAMP levels triggers a Ca<sup>2+</sup>–calmodulin–kinase–kinase–β signaling pathway, leading to the activation of AMPK. In addition, LopesCosta and colleagues [45] have provided some evidence that RSV can correct complex I and IV defects in human fibroblasts via Sirt1- and AMPK-independent mechanisms, which involve estrogen receptor (ER) and estrogen-related receptor alpha (ERRα). These are nuclear receptors co-activated by *PGC-1α* and β, which upregulate mitochondrial pathways [46]. Wenz and colleagues [47] reported that RSV and metformin, a biguanide largely used in diabetes therapy, both stabilize mitochondrial respiratory chain supercomplexes, without increasing mitochondrial protein content in cells.

Finally, the stimulation of the retinoid X receptor-α (RXRα) by retinoic acid has been shown to correct the OXPHOS defects in cybrid cells containing different loads of the 3243A>G MELAS mutation, possibly by increasing the RXRα–*PGC-1α* interaction [48]. Further work is required to determine whether this also applies to other types of mtDNA or nDNA mutations.

## 2.2. Endurance training

Endurance training has also been exploited to trigger mitochondrial biogenesis, and shown to delay the effects of aging in mice [22,49]. In addition to *PGC-1α* activation, endurance training seems to activate *PGC-1β*, as well as AMPK, p38γ MAPK, and the hypoxia inducible factors (HIFs) [50]. Notably, recent data showed that double *PGC-1α* and -1β knockout mice had reduced respiration but normal mitochondrial content and morphology, normal muscle fibers composition and normal endurance performance [51]. This work challenges the central role of *PGC-1* proteins in regulating mitochondrial content but the decrease of respiratory capacity indicates an effect of the system in setting OxPhos proficiency. Irrespective of the molecular mechanism, endurance exercise has been reported as beneficial and safe in patients affected by mitochondrial myopathy [52–54], in muscle-specific *Cox10* knockout mice [55], and in the mtDNA *mutator* mice, where it appears to rescue progeroid aging [49]. Importantly, these beneficial effects were not limited to skeletal muscle but also involved other organs, including the brain.

## 2.3. Scavenging toxic compounds

Pharmacological interventions have been used to modify the course of specific mitochondrial diseases characterized by metabolic blocks in mitochondria, which lead to accumulation of toxic substances. A first example is the use of N-acetylcysteine (NAC) and metronidazole to dump high levels of hydrogen sulfide (H<sub>2</sub>S) characteristic of ethylmalonic encephalopathy (EE) [56]. EE is a devastating, multisystem disease of infancy due to mutations in *ETHE1*, a gene encoding a mitochondrial sulfur dioxygenase (SDO) involved in the disposal of H<sub>2</sub>S. H<sub>2</sub>S is produced by the catabolism of sulfurated amino acids in tissues and by the anaerobic bacterial flora in the large intestine, and in concentrations above nanomolar is highly toxic, leading to profound inhibition of the terminal segment of fatty acids beta oxidation and, more importantly, COX, and direct damage to endothelial lining of small vessels [57]. Accumulation of H<sub>2</sub>S then causes a generalized microvasculopathy and COX deficiency, with multiple organ damage, including brain, skin (with petechial purpura and orthostatic acrocyanosis), skeletal muscle and large intestine. The first step of H<sub>2</sub>S metabolism (Fig. 2)



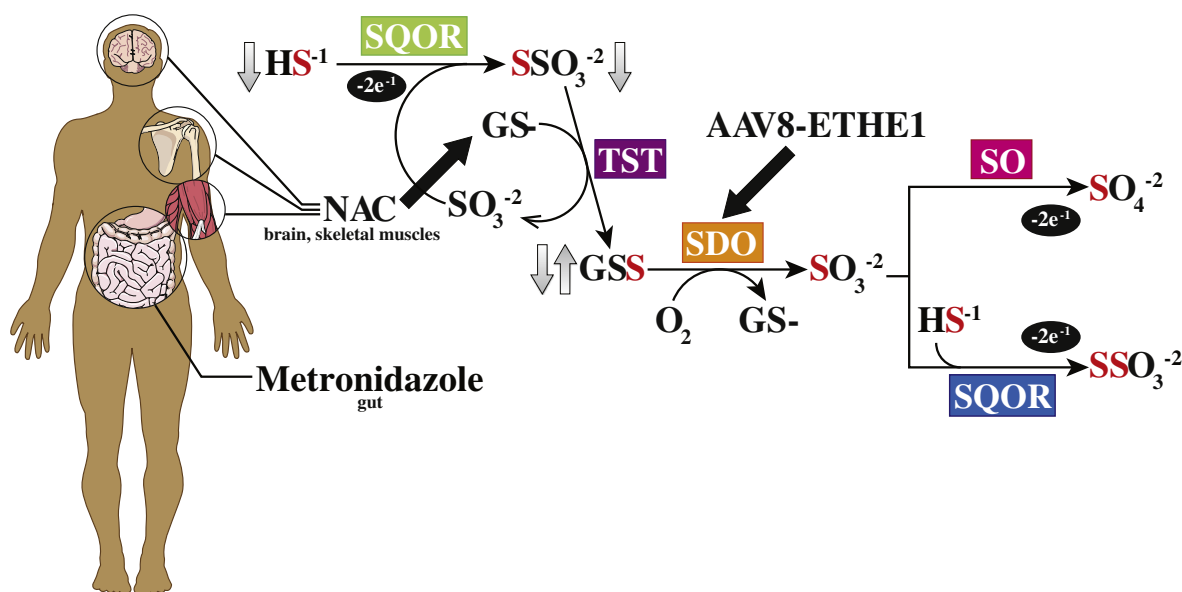


Fig. 2. Metabolism of  $\text{H}_2\text{S}$  and therapeutic interventions.

is catalyzed by sulfide:quinone oxido-reductase (SQOR) to form thiosulfate ( $\text{SSO}_3^{2-}$ ) using sulfite ( $\text{SO}_3^{2-}$ ) as an acceptor for the sulfur sulfane ( $\text{HS}^-$ ) moiety of  $\text{H}_2\text{S}$  ( $\text{H}_2\text{S} + \text{SO}_3^{2-} + 2e^- \rightarrow \text{SSO}_3^{2-}$ ). Thiosulfate is the substrate of thiosulfate:sulfur transferase (TST) [58], which uses reduced glutathione (GSH) to transfer its sulfane sulphur to form glutathione persulfide ( $\text{GSS}^-$ ), whereas the rest of the molecule generates sulfite, which is thus recycled

( $\text{SSO}_3^{2-} + \text{GS}^- \rightarrow \text{GSS}^- + \text{SO}_3^{2-}$ ). The  $\text{GSS}^-$  persulfide is then oxidized by the sulfur dioxygenase activity (SDO) encoded by *ETHE1* to produce sulfite and reduced glutathione ( $\text{GSS}^- + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{GS}^- + \text{SO}_3^{2-}$ ).  $\text{SO}_3^{2-}$  can thus be either oxidized to sulfate ( $\text{SO}_4^{2-}$ ) through the sulfite oxidase (SO) (in the liver) or be recycled through SQOR (in extrahepatic tissues). NAC is a cell-permeable precursor of GSH, which can act as an intracellular  $\text{H}_2\text{S}$  buffer. Metronidazole is an

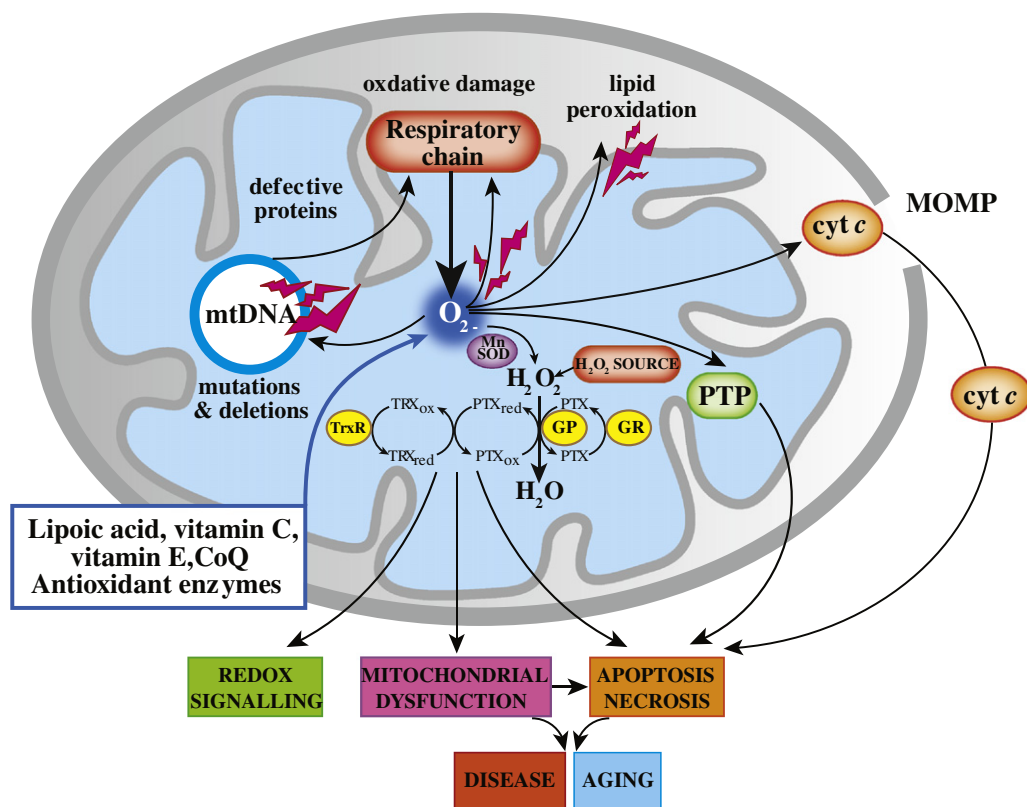


Fig. 3. ROS production and detoxification.

antibiotic specifically active against H<sub>2</sub>S-producing anaerobic bacteria and protozoa. Administration of NAC and metronidazole significantly prolonged the lifespan and clinical conditions of an *Ethe1*<sup>−/−</sup> mouse model, when administered singly or, more effectively, in combination [56]. The same compounds were also effective in a cohort of EE patients, ameliorating some of the clinical hallmarks of the disease, including chronic diarrhea, and diffuse microvasculopathy with acrocyanosis. Some signs of CNS involvement were also improved, leading to increased alertness and wakefulness, and decreased number and duration of epileptic seizures [56].

Reactive oxygen species (ROS), generated as by-products of mitochondrial respiration, play a double role, as they may be potentially harmful but are also important signaling molecules in a number of pathways including adaptation to hypoxia, regulation of autophagy, control of immunological responses, promotion of cell differentiation, and set-up of longevity (Fig. 3) [59,60]. Within mitochondria, highly reactive superoxide (O<sub>2</sub><sup>•−</sup>) is produced at several sites in the matrix and intermembrane space, including the flavin moiety of complex I, the ubiquinone-binding sites in complex III, glycerol 3-phosphate dehydrogenase, the electron transferring flavoprotein:Q oxidoreductase (ETFQOR) of fatty acids and branched-chain amino acid oxidation, and pyruvate and 2-oxoglutarate dehydrogenases [61]. O<sub>2</sub><sup>•−</sup> is rapidly converted into the much less harmful hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by the mitochondrial manganese superoxide dismutase (SOD2) [60]. H<sub>2</sub>O<sub>2</sub> can diffuse through both inner and outer mitochondrial membranes and access the cytosol or can be converted to water by mitochondrial glutathione peroxidases (GPX) or peroxiredoxins (PRX) [60]. On the other hand, superoxide produced in the intermembrane space can exit the mitochondria and be converted into hydrogen peroxide in the cytosol by copper superoxide dismutase (SOD1). Cytosolic H<sub>2</sub>O<sub>2</sub> is believed to be the main form of ROS with signaling function in the cell as it can oxidize protein thiol residues. Its levels are tightly regulated by reduction to water, operated by cytosolic GPXs and PRXs and peroxisomal catalase [60]. Increased ROS production occurs as a consequence of respiratory chain dysfunction due to, for instance, aging [62] or specific OXPHOS defects [63], and may lead to damage of cellular structures, including proteins, lipids and nucleic acids. These observations constitute the rational basis for the use of antioxidants in the therapy of mitochondrial diseases. At the same time, however, ROS can transduce signals in a number of pathways [59]. Cocktails of antioxidant compounds, including lipoic acid, vitamins C and E, and CoQ, have extensively been used in the therapy of mitochondrial diseases for a long time, but no quantitative studies have been carried out in animal models to validate their use. Likewise, randomized double blind trials are still missing to support their efficacy in patients [1,15]. In addition, although a transgenic mouse overexpressing a mitochondrially-targeted catalase shows increased lifespan and resistance to oxidative damage [64], the efficacy of antioxidants in cellular and/or animal models of OXPHOS defects is still controversial. However, two recent papers underline the importance of ROS overproduction in the pathogenesis of cl-related Leigh syndrome [65] and a potential therapeutic target in cl-related disorders in cell models [66].

#### 2.4. Supplementation of nucleotides

Supplementation of deoxyribonucleotides can effectively correct mtDNA depletion in patients' fibroblasts carrying mutations in enzymes involved in the control of the mitochondrial nucleotide and deoxynucleotide pools (e.g. deoxy-guanosine kinase, dGK, and thymidine phosphorylase, TP, encoded by the *DGUOK* and *TYMP* genes respectively).

Likewise, mtDNA depletion has also been corrected in vivo, by treating a *Tymp* knockout mouse model with either dCtd or tetrahydrouridine, an inhibitor of nucleotide catabolism [67–70]. Mutations in human *TYMP1*, encoding TP, are responsible of

mitochondrial neuro-gastro-intestinal encephalomyopathy, MNGIE [71]. MNGIE is a severe, autosomal recessive mitochondrial disorder of early adulthood, characterized by painful gastrointestinal dysmotility causing chronic diarrhea and leading to cachexia, progressive external ophthalmoplegia with mitochondrial myopathy, and severe sensory-motor peripheral neuropathy. Patients usually die of complications due to their critical nutritional status, with an average age at death of 37 years [72]. TP is a cytosolic enzyme catalyzing the first step of thymidine (dThd) and deoxyuridine (dUrd) catabolism. As a consequence of TP dysfunction, MNGIE patients accumulate dThd and dUrd systemically, which ultimately results in imbalances of the mitochondrial pool of deoxyribonucleoside triphosphates (dNTPs) [73]. In fact, increased deoxythymidine triphosphate (dTTP) and decreased deoxycytidine triphosphate (dCTP) have been measured in vitro and in vivo. This dNTP imbalance is mutagenic for mitochondrial DNA (mtDNA), resulting in depletion, multiple deletions, and point mutations accumulating in post-mitotic organs, notably intestinal smooth muscle, skeletal muscle and the nervous system, and cause progressive mitochondrial deficiency and organ failure. Although the mouse model has hardly any clinical sign, it is clearly characterized by markedly abnormal dNTP pools, similar to MNGIE patients.

Promising results were also obtained in a Thymidine Kinase 2 (*Tk2*) H126N knockin mouse reproducing a pathological mutation found in patients. *Tk2* encodes the gene for the mitochondrial thymidine kinase, which phosphorylates thymidine and deoxycytidine pyrimidine nucleosides to generate deoxythymidine monophosphate (dTMP) and deoxycytidine monophosphate (dCMP). Absence of *Tk2* determines an imbalance of dNTP pools leading to mtDNA instability and depletion. The *Tk2* H126N reproduces a human disease characterized by early-onset fatal encephalomyopathy due to mtDNA depletion and multiple RC defects. Treatment with 200 or 400 mg/kg/day leads to increased dNTP concentrations and mtDNA content, rescuing the RC defects, and significantly prolonging lifespan from 13 to 34 days.

#### 2.5. Targeting autophagy

Autophagy (literally “self-eating”) is a physiological pathway aimed at two fundamental and related goals: (i) to recycle energy by degradation of cellular components, and (ii) to warrant quality control of cellular organelles [74–76]. These two goals are achieved through complex processes tailored to selectively eliminate single macromolecules or small organellar portions (microautophagy), or entire organelles that are damaged or supernumerary (macroautophagy), including peroxisomes (pexophagy), endoplasmic reticulum (ER-phagy) and mitochondria (mitophagy). In macroautophagy, the specific target is ultimately engulfed within a double-membrane vacuole called the autophagosome, that eventually gets fused with lysosomes to form the autolysosome, where complete digestion of the organelle components takes place [77]. The specificity of the cargo is determined by specific receptors on the surface of the organelles, targeting them to the pre-autophagosome [78].

Autophagy is regulated by several metabolic sensors, such as growth factors, amino acids and glucose concentrations, and energy status. Anabolic conditions, e.g. high glucose and amino acid availability, activate transduction cascades converging on two main pathways both causing inhibition of autophagy (Fig. 4). In the first pathway, the mammalian target of rapamycin complex 1 (mTORC1) is activated and inhibits autophagy. In the second pathway, which acts independently from mTORC1, cyclic AMP (cAMP) levels are increased, leading to increased inositol-1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>). This causes the release of Ca<sup>2+</sup> from the ER and inhibition of autophagy via Ca<sup>2+</sup>-activated calpains. Conversely, catabolic conditions activate pathways such as the AMPK cascade, and the basic helix–loop–helix leucine zipper transcription factor EB (TFEB), which trigger autophagy. In conditions of low energy (e.g. starvation) TFEB is phosphorylated and consequently migrates to the nucleus, where it promotes autophagic and lysosome

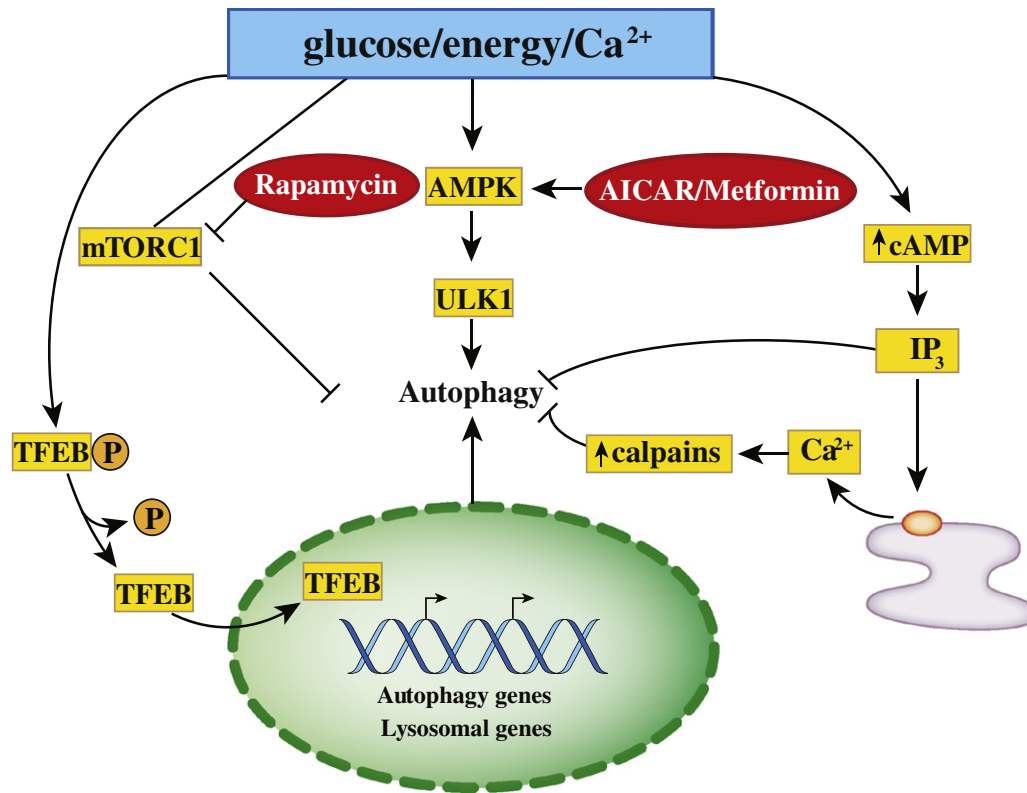


Fig. 4. Regulation of autophagy.

biogenetic programs. AMPK also inhibits mTORC1 and activates UNC51-like kinase 1 (ULK1) complex, a serine/threonine protein kinase, which stimulates the autophagic cascade.

Chronic treatment with the mTOR inhibitor rapamycin, which activates autophagy, significantly delayed both disease progression and fatal outcome of a *Ndufs4*<sup>−/−</sup> mouse which lacks the 18 kDa *Ndufs4* subunit of complex I. Mutations of *NDUFS4* are associated with autosomal recessive, severe infantile Leigh disease in humans and with rapidly progressive, early fatal neurological failure in the *Ndufs4*<sup>−/−</sup> mouse model [79]. Although the underlying mechanism remains partly unexplained, the effect of rapamycin seems to be exquisitely metabolic, as no increase in complex I activity or amount was detected in treated vs. untreated mice. In fact, metabolomic analysis of *Ndufs4*<sup>−/−</sup> brains was hallmarked by accumulation of pyruvate, lactate, and glycolytic intermediates, as well as reduced free amino acids, free fatty acids, nucleotides, and products of nucleotide catabolism, increased oxidative stress markers, and reduced levels of GABA and dopamine. Rapamycin treatment corrected several of these abnormal metabolic biomarkers. However, more investigation is warranted to clarify the underlying mechanism, and to extend it to other models of mitochondrial disease.

## 2.6. Dietary manipulations

Several approaches based on dietary measures have been attempted, with controversial results. Ketogenic diet (KD), i.e. a high-fat, low-carbohydrate diet, has been proposed to stimulate mitochondrial beta-oxidation, and provide ketones, which constitute an alternative energy source for the brain, heart and skeletal muscle. Ketone bodies are metabolized to acetyl-CoA, which enters the Krebs cycle and is oxidized to feed the RC and ultimately generate ATP via OXPHOS. This pathway partially bypasses complex I via increased synthesis of succinate, which donates electrons to the respiratory chain via complex II. Increased ketone bodies have also been associated with increased

expression of OXPHOS genes, possibly via a starvation-like response [80]. Starvation is a stressing condition to the cell, which results in activation of many transcription factors and cofactors (including SIRT1, AMPK, and PGC-1 $\alpha$ ) that ultimately increase mitochondrial biogenesis [80]. KD reduced the mutation load of a heteroplasmic mtDNA deletion in a cybrid cell line from a Kearns–Sayre syndrome patient [81], was shown to increase the expression levels of uncoupling proteins and mitochondrial biogenesis in the hippocampus of mice and rats [82,83], and increased mitochondrial GSH levels [84] in rat brain. These phenomena could contribute to explain the anticonvulsant effects of KD. In a preclinical trial on the *deletor* mouse, KD slowed the progression of mitochondrial myopathy [85]. However, other reports showed that KD can have the opposite effect, and worsens the mitochondrial defect in vivo, for instance in the *Mterf2*<sup>−/−</sup> [86], or the *Mpv17*<sup>−/−</sup> mouse models [87].

Similar to KD, a high fat diet (HFD) was shown to have a protective effect on fibroblasts with complex I deficiency and be effective in delaying the neurological symptoms of the Harlequin mouse, a model of partial complex I defect associated with a homozygous mutation of *AIFM1*, encoding the mitochondrial apoptosis inducing factor [88].

Similar results could in principle be achieved using other compounds that release succinate in mitochondria. An example is triheptaoin, an anaplerotic compound inducing a rapid increase of plasmatic C4- and C5-ketone bodies, the latter being a precursor of propionyl-CoA, which is then converted into succinyl-CoA. Treatment with triheptaoin has been reported to dramatically improve cardiomyopathy in patients with VLCAD deficiency and myopathic symptoms in CPT2 deficiency patients [89,90].

## 2.7. Targeting the PTP

The permeability transition pore is a transient channel deemed to be formed by ATPase dimers [91], which opens upon stress stimuli, such as excessive mitochondrial  $\text{Ca}^{2+}$  uptake, increased ROS, decreased

mitochondrial membrane potential, and low ATP levels. The opening of the PTP leads to complete dissipation of the mitochondrial membrane potential, osmotic swelling of the organelle and ultimately mitochondrial disruption; as a consequence of the release of cytochrome c and other apoptotic triggers, the cell can eventually die. Substantial cell loss or damage may lead to organ failure and disease. Thus, targeting the PTP is a potentially effective strategy to prolong cell survival, slow disease progression, and diminish symptoms severity [92]. Cyclosporine A (CsA) has for long been known to inhibit the PTP through a cyclophilin-D dependent mechanism. CsA has recently been used in patients with Bethlem/Ullrich congenital muscular dystrophy, which are allelic conditions due to mutations in the gene encoding collagen VI. Mitochondrial dysfunction and proneness to apoptosis in skeletal muscle have been documented in both syndromes, and CsA treatment for one month corrected these phenomena in a cohort of five patients [93]. However, while apoptosis plays well-established roles in several pathologies, its contribution to the pathogenesis of primary mitochondrial diseases is not univocally established.

### 3. Molecular approaches to treat mitochondrial diseases

#### 3.1. Targeted re-expression of the mutated gene

Correction of a mutation by expressing the wild-type gene in critical organs has for long been envisaged as the definitive cure for genetic diseases. Although we are still far from having achieved a general strategy for gene replacement in the whole body, large-size organs (e.g. skeletal muscle), or impermeable organs (e.g. brain), several successes have been reported in the last years for a number of genetic diseases, in both preclinical models and patients. In fact, while expression of therapeutic genes through the whole body is still unachievable, and quantitative targeting of skeletal muscle is only feasible in small rodents but not in humans, smaller organs can be targeted by exploiting currently available technologies. In particular the introduction of adeno-associated viral (AAVs) vectors has given new stamina to gene therapy. AAVs belong to the parvoviridae family, which are not associated with any disease in humans or animals, and remain episomic in the cells for prolonged time, thus reducing the risk of insertional mutagenesis [94]. In addition, several serotypes with  $\delta$ -different cellular specificity have been selected, allowing specific targeting of several organs and tissues [95] (Fig. 5).

In the context of mitochondrial disease models, AAV2 was first administered by local injections to correct the myopathy associated with *Ant1*<sup>-/-</sup> mice [96]. More recently, we reported that a recombinant construct expressing human *Ethe1*<sup>wt</sup> could be targeted to the liver using a hepatotropic AAV2/8 serotype. When titers > 10<sup>12</sup> viral genomes/kg were injected in three-week old *Ethe1*<sup>-/-</sup> mice, *Ethe1*-associated SDO activity was completely recovered in liver [57], leading to efficient clearance of H<sub>2</sub>S from the bloodstream. This treatment was associated with significant rescue of the profound COX deficiency due to the inhibitory effect of H<sub>2</sub>S, correction of the other biomarkers of the disease (e.g. high plasma and urine levels of ethylmalonate, lactate and thiosulfate), remarkable clinical improvement and marked prolongation of the lifespan, from a few weeks in untreated animals to over 8 months in AAV-treated littermates [97]. Notably, preliminary data in the same mouse model suggests that administration of the AAV2/8 construct in two doses at P1 and P21 is even more effective, leading to further prolongation of the lifespan to over 1.5 years (Di Meo et al., unpublished).

The same liver-specific AAV2/8 vector has been exploited to treat a mouse model for MNGIE [98]. Although the *Tymp*<sup>-/-</sup> mouse displays hardly any clinical sign, it is characterized by markedly abnormal dNTP pools, similar to MNGIE patients. Intra-venous injection of AAV2/8 particles expressing human wt TYMP (10<sup>12</sup>–10<sup>13</sup> viral DNA/kg) normalized dCTP and dTTP levels in plasma and tissues for up to 8 months of age. This encouraging proof-of-principle result supports the transferability of the AAV2/8 treatment to cure MNGIE patients. The current standard treatment for MNGIE relies on bone marrow transplantation [99,100], which is however burdened by a >50% post-graft mortality due to poor clinical conditions of the recipient patients. Anon-invasive and safe procedure like systemic administration of suitably engineered AAV vectors is clearly more acceptable and can lead to substantial improvement of the otherwise ominous prognosis of this extremely invalidating disorder.

Another potential application of AAV2/8-mediated gene therapy is for correcting liver-specific mitochondrial dysfunction. To demonstrate this, we have used the *Mpv17*<sup>-/-</sup> mouse. *Mpv17* is a small protein of unknown function embedded in the inner mitochondrial membrane, which is mutated in patients affected by hepato-cerebral forms of severe mtDNA depletion syndrome [101], including Navajo neuro-hepatopathy [102]. Similar to the human disease, the mouse model shows profound decrease

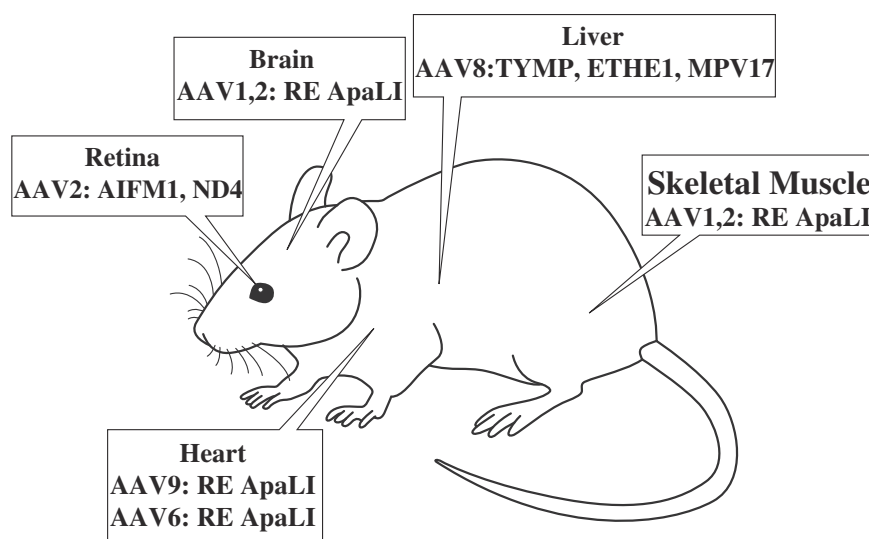


Fig. 5. AAV-based gene therapies in mouse models of mitochondrial diseases.



of mtDNA copy number in the liver, but, in contrast to humans, hardly any clinical phenotype of hepatopathy is detected in standard conditions [103]. However, liver steatosis evolving into cirrhosis associated with fatal liver failure is produced in *Mpv17*<sup>−/−</sup> exposed to KD [87]. We showed that an AAV2/8 viral vector expressing human *MPV17*<sup>wt</sup> fully rescued the mtDNA depletion and prevented the KD-induced cirrhosis in *Mpv17*<sup>−/−</sup> mice, when the treatment was initiated before starting the KD regime, whereas the same treatment significantly delayed but not arrested disease progression when initiated after starting KD [87].

Notably, the recent introduction of new serotypes efficiently and selectively targeting the liver, in particular AAV5, opens the possibility to repeat the injection well after the first administration without incurring in immunological neutralization [104,105].

Finally, an AAV2 vector [106] has also been used to re-express AIF in the eye of the Harlequin mouse, leading to correction of complex I deficiency and long-lasting protection of retinal ganglion cells and optic nerve from degeneration [107].

Taken together, these preclinical results demonstrate the great potential of AAV-mediated gene therapy to combat specific mitochondrial diseases. Nevertheless, a number of issues will need to be addressed in the coming years, including the development of suitable strategies to effectively target extra-hepatic, critical organs such as skeletal muscle, heart and brain. Although some success has been obtained in the treatment of non-mitochondrial myopathies and dystrophies in preclinical models [108–110], their efficacy in humans is still under investigation.

A strategy repeatedly proposed to correcting mtDNA mutations in protein-encoding genes is based on allotopic expression. In this approach, the recoded wild type gene, transfected to the nucleus, expresses a recombinant protein containing a mitochondrial targeting sequence (MTS), to address it to mitochondria. A 3′-UTR signal is usually added, that in yeast serves to target transcripts of mitochondrial proteins to the organelle surface. These transcripts are then translated by a local pool of ribosomes into proteins, which can promptly be imported into the organelle. This approach has been attempted in fibroblasts carrying mutations in ND1, ND4 and ATP6 genes [111–113] and in a rat model of LHON [114]. Taken together, the results from these experiments are very controversial, as conflicting data have been obtained by different groups on the ability of recoded, mitochondrially targeted mtDNA gene products to be effectively imported and correctly inserted within the respiratory chain complexes [115]. In particular, allotopically expressed ND6 gene, recoded according to the universal genetic code, failed to be imported into mitochondria, remaining stuck onto the OMM. The correction of the complex I defect reported in these experiment was later deemed to be a spurious result due to selection of spontaneous revertants in the cell culture [115]. Nevertheless, two open-label clinical trials based on AAV-mediated allotopic expression of mtDNA genes for LHON are currently recruiting patients (<https://clinicaltrials.gov>). Likewise, a therapeutic strategy for LHON, based on the use of an AAV2 construct has been proposed to express the wild type ND4 gene in LHON mutant cybrids and in a transgenic rat model of LHON. The AAV2 capsid protein VP2 has been engineered by adding an MTS in order to promote the internalization of the viral particle into mitochondria [116]. Again, convincing demonstration that the full viral particle is in fact translocated within mitochondria and its ability to properly express the therapeutic gene is, in our opinion, lacking; these controversial results have not been replicated by others.

The multiploidy organization of mtDNA, its confinement within an almost impermeable double-membrane barrier, and the virtual absence of homologous recombination, are formidable hurdles against the direct, controlled manipulation of this genome for therapeutic intervention. Nevertheless, several approaches have been proposed to this aim, and some are giving promising results. A first strategy was based on the use of protein nucleic acids (PNAs) as an “antigenomic” device [117,118]. PNAs are synthetic DNA-like molecules in which the

pyrimidine and purine residues are linked to an aminoethyl (pseudopeptide) backbone. Since these molecules are not charged at physiological pH, a PNA binds its complementary DNA with greater affinity than natural nucleic acids, so that PNA–DNA hybrids are more stable than DNA–DNA hybrids. PNAs complementary to either the mtDNA stretch containing the 8344A>G MERRF mutation in mt-rRNA<sup>Lys</sup> [5], or the breakpoint junction associated with the mtDNA common deletion, were shown to be imported into mitochondria, where they inhibited the replication of mutant but not wild type mtDNA; however no such effect was demonstrated in cell lines [119]. In another approach, a synthetic N-terminal signal was used to introduce oligonucleotides complementary to mtDNA into the mitochondrial matrix. Oligonucleotides were annealed to complementary PNAs and the hybrid molecule was selectively imported into the mitochondrial matrix, but its antigenomic effect was not demonstrated [120].

Recently, an alternative approach has been used to target allotopically expressed tRNAs and mRNAs to the mitochondria. This approach takes advantage from the observation that RNase P, a ribonucleoprotein involved in the processing of mitochondrial transcripts, is imported into mitochondria through a specialized system (PNPase) that specifically recognizes its RNA component (H1 RNA). By fusing the gene of interest with a 20-ribonucleotide stem-loop sequence from the H1 RNA, some evidence was provided in support of correction of mt-tRNA and COII gene mutations in cell lines [121,122]. These findings deserve further investigation and independent confirmation.

### 3.2. Manipulating mtDNA heteroplasmy

As pathogenic mutations of mtDNA are often heteroplasmic, and behave as “recessive-like” mutations, suitable therapeutic intervention can be envisaged, aimed at eliminating or reducing the amount of mutated DNA below the threshold at which the disease manifests. This result has been achieved in cellular models by targeting to mitochondria recombinant restriction endonucleases [123–125], zinc finger-endonucleases [126] or TALENs [127]. Mitochondrially targeted restriction enzymes have been used in a variety of systems to induce a shift in heteroplasmy. For instance, the 8399T>G NARP mutations forms a unique CCCGGG restriction site in mtDNA specific to the restriction endonuclease *SmaI* (the wild type sequence is CCCGTG). A mitochondrially targeted recombinant *SmaI* variant was in fact able to substantially decrease the 8399T>G mutation load in heteroplasmic mutant cybrids. This was followed by repopulation of cells with wild-type mtDNA, restoration to normal of mitochondrial membrane potential and increase of intracellular ATP levels [128,129]. The *PstI* endonuclease, whose restriction site is present in human and mouse but not rat mtDNA, was targeted to human mitochondria, where it degraded mtDNA, and determined a shift towards the rat haplotype in a hybrid cell line harboring both mouse and rat mtDNA [125]. Subsequent work has demonstrated the efficacy of this approach in NZB/BalbC heteroplasmic mice in which AAV1,2 vectors expressing a mitochondrially-targeted restriction endonuclease *ApaI* were injected locally into muscle or brain [130]; other vectors expressing the same endonuclease were administered by i.v. infusion to target specific organs, such as the liver (with adenovirus) or the heart (with AAV6) [123,131]. In addition, an AAV9 vector expressing the mitochondria-targeted *ApaI* was also used to markedly shift mtDNA heteroplasmy in skeletal and cardiac muscle of neonate mice harboring two mtDNA haplotypes that differed for the presence or absence of a unique *ApaI* restriction site (GTGCAC) [124].

In practice, this approach can be used therapeutically only if a unique restriction site is created by an mtDNA mutation, as in the case of the 8993T>G NARP, an exceptionally rare event. However, the recent development of zinc-finger nuclease and TALEN technologies can offset this limitation. Zinc finger nucleases (ZFNs) are chimeric enzymes in which the modular Cys<sub>2</sub>His<sub>2</sub> zinc finger DNA-binding domains present

in numerous transcription factors are conjugated to the C-terminal catalytic subunit of the type II restriction enzyme *FokI* [132, 133]. Each zinc finger domain recognizes three nucleotides, so that appropriate arrangements of the zinc finger modules permit to target virtually any DNA sequence for nucleolytic cleavage. ZFN can be targeted to mitochondria by adding a suitable MTS at the N-terminus. Likewise, transcription activator-like effector nucleases (TALEN) exploit DNA-binding domains of the *Xanthomonas* bacteria composed of 33–35-amino-acid repeats, each recognizing a single base pair, fused with the *FokI* nuclease. Again, TALENs can be targeted to mitochondria via an N-terminal MTS (MitoTALENs).

MitoTALENs [127] have been proven to eliminate heteroplasmic mutant mtDNA in cybrid cells carrying either the m.8483\_13459del4977 common mtDNA deletion [134–136] or the m.14459G>A LHON/Dystonia mutation in the MT-ND6 gene [137]. In both cases, a transient decrease in total mtDNA levels occurred, followed by repopulation with wild type mtDNA up to normal values.

Likewise, mitochondrially targeted ZFNs (mtZFNs) were successfully used in heteroplasmic cybrids to cleave mtDNA harboring either the heteroplasmic m.8993T>G NARP mutation [6] or the common deletion. As for TALENs and restriction enzymes, mtZFNs led to a reduction in mutant mtDNA haplotype load, and subsequent repopulation of wild-type mtDNA, associated with restoration of mitochondrial respiration [126].

### 3.3. Stabilizing mutant mt-tRNA

More than 50% of the mtDNA mutations are localized in tRNA genes, leading to a wide range of syndromes, such as MELAS or MERRF. Aminoacyl-tRNA synthetases (aaRSs) are ubiquitously expressed, essential enzymes performing the attachment of amino acids to their cognate tRNA molecules as the first step of protein synthesis [138]. Several lines of evidence in yeast and human cell lines indicate that overexpressing cognate mt-aaRS can attenuate the detrimental effects of mt-tRNA point mutations [139–142]. For instance, overexpression of mt-leucyl-tRNA synthetase (mt-LeuRS) corrects the respiratory chain deficiency of trans-mitochondrial cybrids harboring the MELAS mutation in the mt-tRNA<sup>Leu(UUR)</sup> gene (*MTTL1*) [138,141]. Likewise, overexpressing the cognate mt-valyl-tRNA synthetase (mt-ValRS) restored, at least in part, steady-state levels of mutated mt-tRNA<sup>Val</sup> in cybrid cell lines [142]. Finally, constitutive high levels of mt-isoleucyl-tRNA synthetase (mt-IleRS) were shown to be associated with reduced penetrance of the homoplasmic m.4277T>C mt-tRNA<sup>Ile</sup> mutation, which causes hypertrophic cardiomyopathy [143]. In addition, experiments in yeast and human cells have shown that the overexpression of either human mt-LeuRS or mt-ValRS was able of rescuing the pathological phenotype associated with mutations in both the cognate and the non-cognate mt-tRNA. A region in the carboxy-terminal domain of mt-LeuRS was found necessary and sufficient to determine this phenomenon, probably via a chaperone-like stabilizing effect [144,145].

An alternative approach to the same issue was based on the observation that in yeast some tRNAs were encoded in the nuclear genome and imported into the mitochondria. So, tRNA mutations in mtDNA may in principle be complemented by expressing a xenotopic nDNA-encoded yeast mitochondrial tRNA from the mammalian nucleus. This approach has been attempted for the treatment of human cells harboring the tRNA<sup>Lys</sup> nucleotide 8344A>G mutation using the yeast tRNA<sup>Lys</sup> nDNA gene [122,146,147]. This partially restored the mitochondrial dysfunction associated with the mitochondrial protein-synthesis defect. Similarly, a *Leishmania*-mitochondrial RNA import complex has been exploited to introduce the human cytosolic tRNA<sup>Lys</sup> into human cybrids harboring the tRNA<sup>Lys</sup> 8344A>G mtDNA mutation by a caveolin-1-dependent pathway, obtaining a significant restoration of mitochondrial function [148].

These findings, however, are still controversial and need confirmation from independent labs.

### 3.4. Targeting fission and fusion

Mitochondria are highly dynamic organelles whose shape and mass are finely tuned by the activity of pro-fusion proteins, such as mitofusin 1 (MFN1), MFN2 and optic atrophy protein 1 (OPA1) and pro-fission proteins, such as dynamin-related protein 1 (DRP1) and mitochondrial fission 1 protein (FIS1) [78,149]. Alterations in the genes encoding these complex machineries lead to disease in humans. For instance, mutations in *OPA1* are associated with autosomal dominant optic atrophy [150] and mutations in *MFN2* cause Charcot-Marie-Tooth disease type 2A [151]. In addition, disruption of *Mfn1* and *Mfn2* in the skeletal muscle of the *POLG*<sup>D257A</sup> mutator mouse leads to striking worsening of the phenotype, due to accumulation of mtDNA mutations, suggesting that the physiological balance between fission and fusion protects the integrity of mtDNA through continuous mixing of mtDNA pools [152]. Two additional observations are relevant in this context. First, overexpression of *Opa1*, a multitasking GTPase involved in shaping mitochondrial cristae and promoting fusion of the inner mitochondrial membrane, has been shown to increase respiratory efficiency by stabilizing the respiratory chain supercomplexes [153]. Second, some compounds affecting fission and fusion have been identified, such as the Drp1 inhibitor MDIVI-1 and M1-hydrazine that probably promotes fusion by acting on *Mfn* or *Opa1*. However, the therapeutic potential of these compounds for mitochondrial diseases has still to be proved.

### 3.5. Bypassing the block of the respiratory chain

An emerging concept in mitochondrial medicine is the possibility to by-pass the block of OXPHOS due to mutations affecting the RC complexes by using the “alternative” enzymes NADH dehydrogenase/CoQ reductase (Ndi1) and CoQ/O2 alternative oxidase (AOX).

These are single-peptide enzymes, located in the mitochondrial inner membrane, which transfer electrons to (Ndi1) and from (AOX) CoQ, without pumping protons across the membrane. Ndi1 substitutes complex I in yeast mitochondria. AOX is an alternative electron transport system present in lower eukaryotes, plants and several invertebrates that by-passes the complex III + IV segment of the respiratory chain. Expression of these proteins is well tolerated in mammalian cells [154], flies and mice [155] and has successfully been exploited to by-pass complex I or complex III/IV defects in human cells [156,157] and *Drosophila* models [158–160]. The therapeutic mechanism is based on the capacity of these enzymes to restore the electron flow through the quinone pool, thus preventing accumulation of reduced intermediates and oxidative damage [161]. However, this is not accompanied by restoration of proton translocation across the inner mitochondrial membrane, and does not directly increase ATP production. Nevertheless, the restoration of the electron flow can reactivate the unaffected RC complexes, thus indirectly promoting the rebuilding of the proton gradient and the reactivation of OXPHOS. AOX-expressing mice have recently been created and shown to be viable and fertile [155], thus opening the possibility to test whether this approach is amenable in a mammalian organism, using suitable mouse models of complex III or IV deficiency.

### 3.6. Somatic nuclear transfer

Given the difficulty of manipulating mtDNA and the uncertainties of genetic counseling for mtDNA mutations, prenatal or pre-implantation genetic diagnosis is nowadays the best option available to women carrying pathogenic mtDNA mutations. However, these techniques can only be applied to subjects with low levels of mtDNA mutations in oocytes and are technically challenging. Recent technical improvements in non-human primates [162] and non-viable human embryos [163,

[164] have paved the way to replace the mutated maternal mtDNA with that obtained from a healthy woman, by transferring either the spindle-chromosomal complex of mature oocytes, or the pronuclei during the pre-zygotic stage of fertilized egg. Both techniques have been refined in order to minimize the amount of mutant mtDNA carried over into the recipient ooplasm. A child born by these procedures will carry the nuclear genes of the affected mother (and healthy father) but the healthy mitochondrial genes of the donor (see also [165] for a very recent summary of the ongoing debate on this important topic).

#### 4. Conclusions

Mitochondrial diseases are amazingly complex and its biology has so far prevented the development of effective therapy for most of them. Nevertheless, the last few years witnessed numerous attempts to significantly modify the phenotype in cellular and animal models by using either disease-specific or wide-spectrum strategies applicable to several disorders. The wealth of knowledge accumulated in over 25 years of intensive studies aimed at elucidating the genetic causes and the pathogenic mechanisms of mitochondrial diseases has driven these first “proof of concept” successes that now need to be translated and tested on patients. In addition, mitochondrial dysfunction is nowadays recognized as central in several medical conditions, including diabetes, inflammation, cancer and neurodegeneration; this will certainly have a synergistic effect to expand our knowledge on the pathomechanisms underlying both primary and secondary mitochondrial impairment and to prompt the development of more effective, evidence-based therapeutic approaches.

#### Transparency Document

The [Transparency Document](#) associated with this article can be found, in the online version.

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